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High genetic diversity and variability of bacterial communities associated with the sandhopper *Talitrus saltator* (Montagu) (Crustacea, Amphipoda)

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ABSTRACT

The microbiome present in individuals of *Talitrus saltator* belonging to seven populations distributed along the Tuscan coast (Italy) was assessed by using Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis of amplified 16S rRNA genes. *Talitrus saltator* is one of the key species of the damp band of European sandy beaches and despite of the large interest on animal-associated bacteria, only a few and preliminary data were present. Results showed a high diversity of the microbiome, composed mainly by members of *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacillales* and *Clostridiales* classes. The microbiome fingerprints were highly variable among individuals, even from the same populations, the inter-individual differences accounting for 88.7% of total fingerprint variance. However, statistically significant population-specific microbiome signatures were detected, and accounted for the remaining 11.3% of total fingerprint variance. These population-specific differences were mainly attributed to sequences from members of known host-associated bacteria such as *Gammaproteobacteria* and *Betaproteobacteria*, *Cytophagia* and *Spirochaetia*. This study showed the high complexity of the microbiome associated with an amphipod species and on the inter-individual microbiome variation with potential importance for understanding amphipod trophic and ecologic processes.

1. Introduction

Talitrid amphipods, one of the main components (in terms of biomass) of the damp band of sandy beaches, play an important role in the energy flow within the sandy beach ecosystem because they feed on organic matter of marine and terrestrial origin and provide nourishment for many species of beetles, fishes, birds and mammals (Olabarria et al., 2009). Talitrid amphipods constitute the main animal biomass in sandy beaches and play an important role in the supralittoral environment (Griffiths et al., 1983; Wildish, 1988; McLachlan and Brown, 2006). The sandhopper *Talitrus saltator* is a well-established model species for biomonitoring human pressure and pollution on sandy beaches (Barca-Bravo et al., 2008; Ugolini et al., 2004, 2005, 2012; Ungherese et al., 2010a,b, 2012) and for ecology and behavior (Pardi and Papi, 1953; Wildish, 1988; Ugolini et al., 1999; Ugolini, 2003, 2006). Despite their key ecological relevance for carbon cycling on the damp band of sandy beaches, to date there are very limited reports on *T. saltator*-associated gut microbial flora (Nuti et al., 1971; Martineti et al., 1995). In contrast, several reports on Crustacea-associated microorganisms are present, but mainly linked to health and aquaculture issues (Small and Pagenkopp, 2011; Wang, 2011) or to the presence of *Wolbachia* infections (Cordaux et al., 2012). There are no investigations on the total microbial communities carried by Crustaceans, despite the fact that in recent years, invertebrate microbiology is attracting more attention for its implication in biocontrol (Natrah et al., 2011) and animal–microbe interaction studies (Muller et al., 2008; Olson and Kellogg, 2010; Goffredi, 2011). Moreover, despite several studies concerning the microflora of particular organs (e.g. gut or reproductive organs) (Crotti et al., 2010; Hamdi et al., 2011), as well as the presence and effect of *Wolbachia* infections (Ben Nasr et al., 2010; Cordaux et al., 2012), no data were reported about the variation of microbial communities among single individuals, nor about possible population-specific microbial communities.

This work aimed to answer two key basic questions on the ecological interactions of dump band amphipods, using *Talitrus saltator* as model species: 1) What is the composition, in taxonomic terms, of the bacterial community associated with single individuals and populations of *T. saltator*?; 2) Is there individual or population-specific differentiation of the bacterial community? To

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address these questions the richness and the variability (both animal-by-animal and population-specific) of the bacterial communities associated with the sandhopper *T. saltator* were characterized by using Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis (Liu et al., 1997; Mengoni et al., 2007) of amplified 16S rRNA genes, to provide a first insight into the microbiology and microbial ecology of this key species of sandy beaches.

2. Materials and methods

2.1. Sampling and DNA extraction

Adult individuals of *Talitrus saltator* were collected from seven populations (FA, AF, AM, RS, FM, C, P, Fig. 1), on the Tuscan coast (Italy), previously characterized in a population genetic analysis (Ungherese et al., 2010b). Live animals were immediately transported to the laboratory, surface cleaned and conserved at −80 °C. From 3 to 7 animals were analyzed per population for a total of 30 animals. Single animals were surface-cleaned by washing with sterile distilled water to remove dust and sand particles. Total DNA was extracted from animals as previously described (Ungherese et al., 2010b), using a commercial kit (Macherey–Nagel). DNA was quantified spectrophotometrically and stored at −20 °C prior to use.

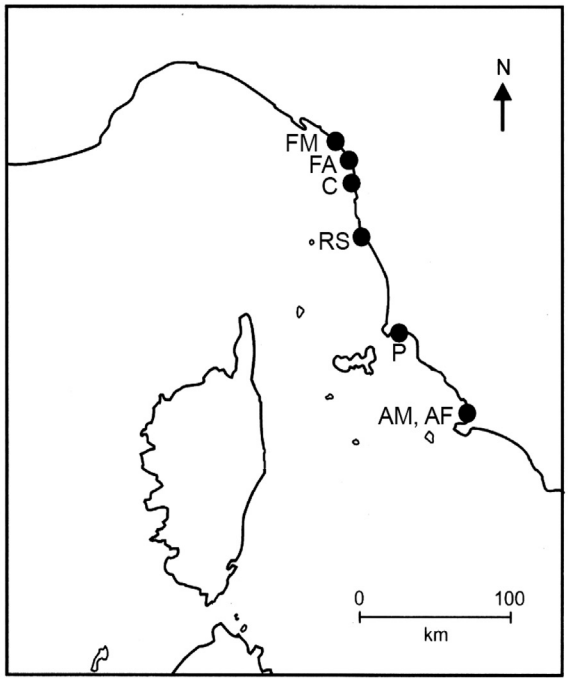
2.2. T-RFLP profiling

T-RFLP was carried out as previously described (Mengoni et al., 2009; Pini et al., 2012) using 799f/pHr primer pair, which doee not target chloroplast and mitochondrial 16S rRNA gene, in order to avoid amplification of 16S rRNA from animal DNA and from the ingested food (*Posidonia oceanica* and others vegetable/organic debris). Purified amplification products were digested separately with restriction enzymes *Alu*I and *Taq*I and digestions were resolved by capillary electrophoresis on an ABI310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using LIZ 500 (Applied Biosystems) as size standard. T-RFLP analysis was performed, as previously reported, on two technical PCR replicates from each DNA extract as previously reported (Mengoni et al., 2005). Only peaks present in both duplicate runs were considered for successive analyses.

2.3. Statistical analyses

Chromatogram files from automated sequencer sizing were imported into GeneMarker ver. 1.71 software (SoftGenetics LLC, State College, PA, USA), by filtering with the default options of the module for AFLP analysis. Peaks (Terminal-Restriction Fragments,

A)



B)

Site	Geographical coordinate	Organic carbon (%)	Lipid content (%)
FM Fiume Morto	43°44'55" N; 10°16'31" E	0.04	14.2
FA Fiume Arno	43°40'55" N, 10°16'52" E	0.05	13.8
C Calambrone	43°34'56" N; 10°17'56" E	0.08	11.7
RS Rosignano Solvay	43°22'24" N; 10°26'23" E	0.74	11.2
P Piombino	42°57'07" N; 10°34'11" E	0.05	13.9
AM Albegna mare	43°30'36" N; 11°11'30" E	n.d.	n.d.
AF Albegna fiume	42°30'16" N; 11°11'41" E	0.18	7.0

Fig. 1. Sampled populations of *T. saltator* along the Tuscan coast (Italy). A) Map of the sampling sites. B) Codes of sites, geographical coordinates, organic carbon content of sandy sediments and total lipid content of animals (data from Ungherese et al. (2012)) are reported.

T-RFs) above 50 fluorescence units and whose size ranged from 35 to 500 nt were considered for profile analysis. Taxonomic interpretation of T-RFs was done by MiCA web tool (http://mica.ibest.uidaho.edu/) (Shyu et al., 2007) and MiCA results were then analyzed and plotted by using Krona (Ondov et al., 2011). Tough MiCA is performing a matching of the TRFs size/primer pairs/restriction enzyme combination with the 16S rRNA gene sequences deposed in the Ribosomal Database (Cole et al., 2009), thus allowing only a "putative" taxonomic assignment (no direct sequences matching), it has been proved as a reliable tool for the taxonomic description of a bacterial community (see for examples (Mengoni et al., 2009; Trabelsi et al., 2011; Bell et al., 2012; Febria et al., 2012; Horz et al., 2012)).

Statistical analyses were performed on a binary matrix obtained by linearly combining data from the two restriction enzymes as previously reported (Mengoni et al., 2009). UPGMA (Unweighted Pair Group Method with Arithmetic mean) clustering of the binary vectors and nonmetric Multidimensional Scaling (n-MDS) were computed on a Jaccard similarity matrix by using the modules present in PAST (Hammer et al., 2001). To test the distribution of the variance of T-RFLP profiles within *Talitrus saltator* populations and among populations and sites, Analysis of Molecular Variance (AMOVA) (Excoffier et al., 1992) was applied using Arlequin 3.5.1.2 software (Excoffier et al., 2007). Pairwise F_{ST} distances (Slatkin, 1995) between T-RFLP profiles, computed with Arlequin 3.5.1.2, were used to infer a Neighbor-Joining dendrogram with the software MEGA5 (Tamura et al., 2011) (Table 2). As previously reported (Mengoni et al., 2009; Pini et al., 2012), AMOVA and pairwise F_{ST}, originally developed to measure the differentiation among populations, were here applied as an estimator of bacterial community differentiation. Mantel's test of correlation between Jaccard distance matrices obtained from T-RFLP profiles and from genotypic fingerprinting of the same animals (Ungherese et al., 2010b) was performed by using Zt software (Bonnet and Van de Peer, 2002).

3. Results

3.1. T-RFLP bacterial community diversity of Talitrus saltator

The T-RFLP profiling of 30 *Talitrus saltator*-associated bacterial communities with two restriction enzymes (*Alu*I and *Taq*I) yielded a total of 56 polymorphic T-RFs, which allowed fingerprinting each single animal-associated bacterial community. Fig. 2a reports the number of Terminal-Restriction Fragments (T-RFs) obtained from each single animal. Values ranged from 7 to 31 T-RFs per animal. The sharing of T-RFs among individuals was relatively low (Fig. 2b), with most of the T-RFs being present in less than 20% of individuals.

The molecular diversity of bacterial communities averaged over the seven *Talitrus saltator* populations is reported in Table 1. Values of the mean number of T-RFs ranged from 8.8 (AF) to 18.8 (FA), with no significant differences among populations. It is of note that when considering the mean number of pairwise differences among T-RFLP profiles within the same population (informative over the differences among the bacterial communities from animals belonging to the same population), the populations AM and AF showed the lower values (8.5 and 7.2, respectively) over the whole

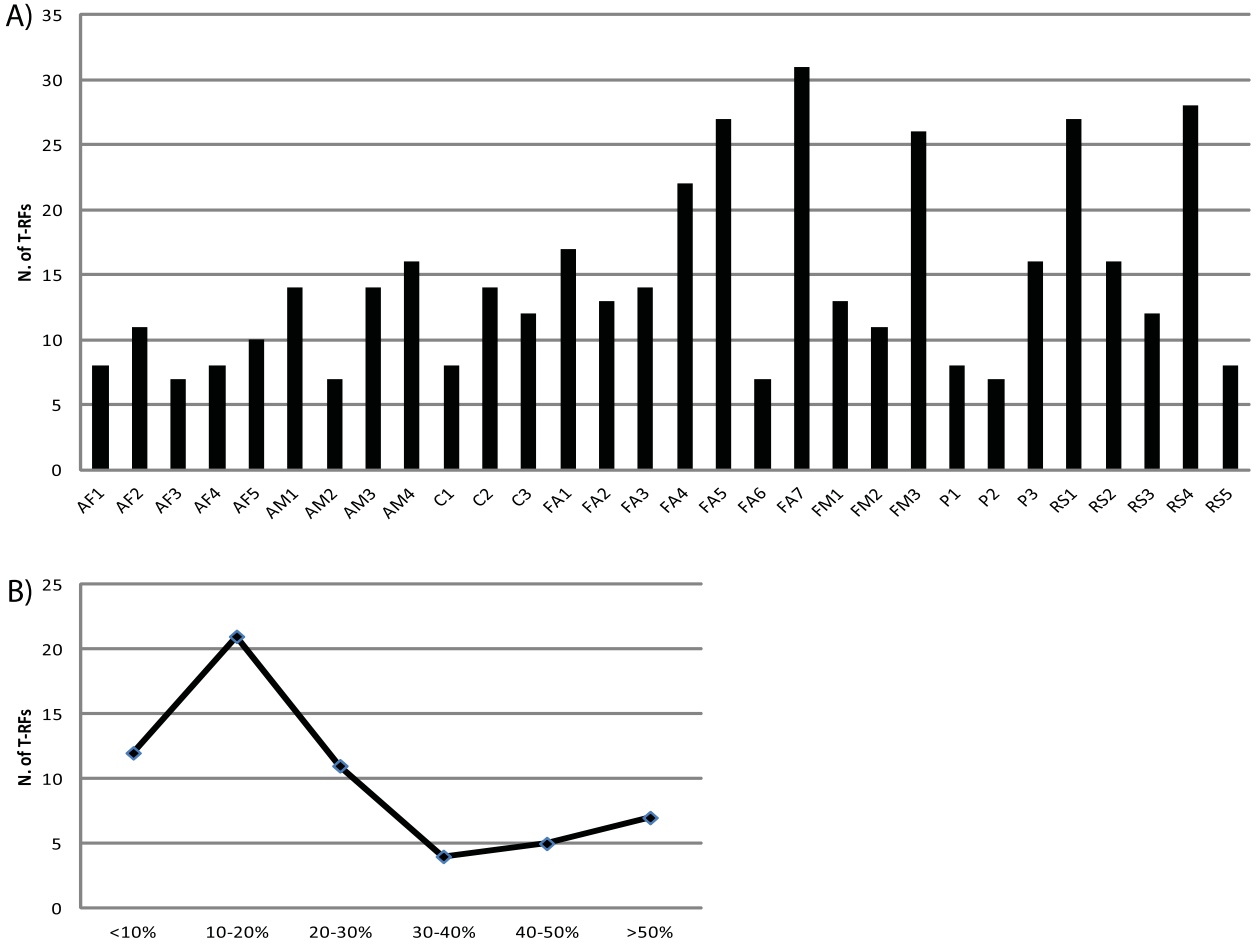


Fig. 2. Bacterial community diversity. A) The number of T-RFs for each animal is displayed. Codes for populations are those reported in the legend of Fig. 1. B) Sharing of T-RFs in the dataset. The number of T-RFs present in different classes of sharing is reported. Codes are those reported in the legend of Fig. 1.

Table 1
Molecular diversity index (mean number of pairwise differences) for bacterial communities associated with seven *T. saltator* populations.*

Populations	Mean n. TRFs	Mean n. of pairwise differences
FA	18.7 ± 8.4	20.6 ± 11.9
FM	12.0 ± 1.0	16.0 ± 12.3
C	11.3 ± 3.0	12.6 ± 9.8
RS	18.2 ± 8.9	21.4 ± 13.3
P	10.3 ± 4.9	12.6 ± 9.8
AM	12.7 ± 3.9	8.5 ± 5.9
AF	8.8 ± 1.6	7.2 ± 4.7
Mean over all populations	13.3 ± 6.8	14.1 ± 9.7

* The mean number of TRFs and the mean number of pairwise differences of T-RFLP profiles are shown for each *T. saltator* populations. ±, standard deviation.

dataset, tough values are not significantly different with respect to the other populations. Moreover, a Spearman test of correlation run on the number of molecular diversity of the community and organic carbon content of sediments and lipid content of animals did not show any significant correlation between community diversity data and lipid content or organic carbon content data (Table S1).

The taxonomic assignment of T-RFs over the Ribosomal Database with MiCA allowed us to attribute the 27 and 29 T-RFs from *Alu*I and *Taq*I restriction digestions to different taxonomic phyla and classes. The taxonomic representation obtained by the two restriction enzymes is shown in Fig. 3. A large part of the TRFs were attributed to the phylum *Proteobacteria*, and in particular to the classes of *Gammaproteobacteria* (especially for *Taq*I profiles, Fig. 3b), *Alphaproteobacteria* and *Betaproteobacteria*. For the phylum *Firmicutes*, sequences from both *Bacillales* and *Clostridiales* were most similar to our TRFs. Interestingly, several groups known to have strains host-associated were retrieved, as *Burkholderia*, *Bacillus*, *Clostridium*, *Enterobacter*, *Stenotrophomonas* etc., which may harbor genes important for the digestive processes (e.g. cellulases from clostridia).

3.2. Variance of bacterial community among Talitrus saltator individuals and populations

To evaluate the similarities of the bacterial community in different *Talitrus saltator* individuals and the relationships between populations, non-Metric Multidimensional Scaling (nMDS), UPGMA clustering and AMOVA were carried out (Fig. 4a and b). Individuals from the same population formed some clustering (e.g. FA and AM), but no strict population clustering was observed.

Table 2
Analysis of Molecular Variance (AMOVA) for T-RFLP profiles of *T. saltator*-associated bacterial communities.*

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P*-value
Among populations	6	69.450	0.95959	11.30	<0.003
Within populations	23	173.283	7.53406	88.70	<0.0001
Total	29	242.733	8.49365	$F_{ST} = 0.11298$	

* AMOVA was performed with T-RFLP profiles from all seven populations included in a single group (two hierarchical levels). The total variance observed was attributed to the two hierarchical partitions: first lane, among populations; second lane, within populations (among single individuals within populations). Data show the degrees of freedom (d.f.), the sum of squared deviation, the variance component estimate, the percentage of total variance contributed by each component, the F_{ST} statistics (Fixation index), and the probability of obtaining a more extreme component estimate by chance alone (*P*). *P*-values were estimated computing 10,000 permutations.

Interestingly, n-MDS grouped together individuals from the two closest populations (AM and AF). To statistically test the differentiation between populations Slatkin's pairwise distances (derived from pairwise F_{ST}) were computed and a Neighbor-Joining dendrogram was drawn from them (Fig. 4c). Results showed indeed a grouping of the T-RFLP profiles of populations partially in agreement with geographical separation (with the relevant exception of the P population). AMOVA (Table 2) showed that populations are differentiated in terms of T-RFLP bacterial community profiles (11.30% of among population variance), even if different animals within the same population retained the highest level of differences in their bacterial community profiles (88.70%). A grouping of populations in a northern (FA, FA, C, RS) and in a southern group (P, AM, AF) resulted in an appreciable level of variance for this partition (9.61% among the northern and the southern group), but the *P*-value was at the threshold ($P < 0.052$) for significance. This somewhat geographically-related pattern warrants more investigation to elucidate the involvement of sandy beaches bacterial communities in the sandhopper microbiome. A survey of substratum bacterial communities and their possible biogeography is needed to ecologically evaluate sandhopper population differences and relationships detected by bacterial community fingerprinting.

To more deeply investigate possible correlations between the *Talitrus saltator* microbiomes (as T-RFLP profiles) and their genetic and geographic separation a Mantel's test was performed on the Jaccard similarity matrices obtained by T-RFLP (this work) and by ISSR (Ungherese et al., 2010b) on the very same animals. Results obtained showed indeed a low correlation value ($r = 0.175$, $P < 0.01$), indicating that bacterial community composition is not directly influenced by host genotype. However, a locus-by-locus AMOVA sorted out 13 out of 59 T-RFs being unevenly distributed among populations ($P < 0.05$), thus accounting for the detected population differentiation, as previously reported (Fig. 4c). These thirteen T-RFs were then taxonomic assigned with MiCA, allowing to associate microbiome population differences to bacterial groups. Results obtained for *Taq*I-derived T-RFs (6 T-RFs matching Ribosomal Database out 10 T-RFs) and *Alu*I-derived T-RFs (3 T-RFs matching Ribosomal Database out 3 T-RFs) showed that (Table 3) differentiation was due to T-RFs matching sequences from members of *Gammaproteobacteria* and *Betaproteobacteria*, but also matches with members of classes *Spirochaetia*, *Firmicutes*, *Sphingobacteriia*, *Deltaproteobacteria*, *Gemmatimonadetes* were present. The T-RF at 59 nt after *Taq*I digestion was attributed to sequences from very different classes (*Clostridia*, *Flavobacteriia*, *Alphaproteobacteria*, *Nitrospira*).

4. Discussion

In recent years several efforts have explored the biodiversity and understanding the functional role of microbial communities associated with organs and tissues of higher organisms (both plants and animals). The microbiomes carried by higher organisms have an important role in determining the health status of the organism (Hamdi et al., 2011; Kinross et al., 2011), its trophism, and ecological interactions. Recent examples include the human gut microbiome (Arumugam et al., 2011; Schloissnig et al., 2012), as well as the giant panda gut microbiome (Zhu et al., 2011) or the plant endosphere (Pini et al., 2012). These works showed that the microbial (bacterial) diversity harbored by higher organisms is in a balance between selection (i.e. certain bacterial taxa are chosen by or are preferentially associated with the higher organism) and chance (i.e. other taxa are occasionally or not always present within the higher organism's microbiome), rendering not obvious direct cause-effect studies and microbe-plant as well as microbe-animal association studies.

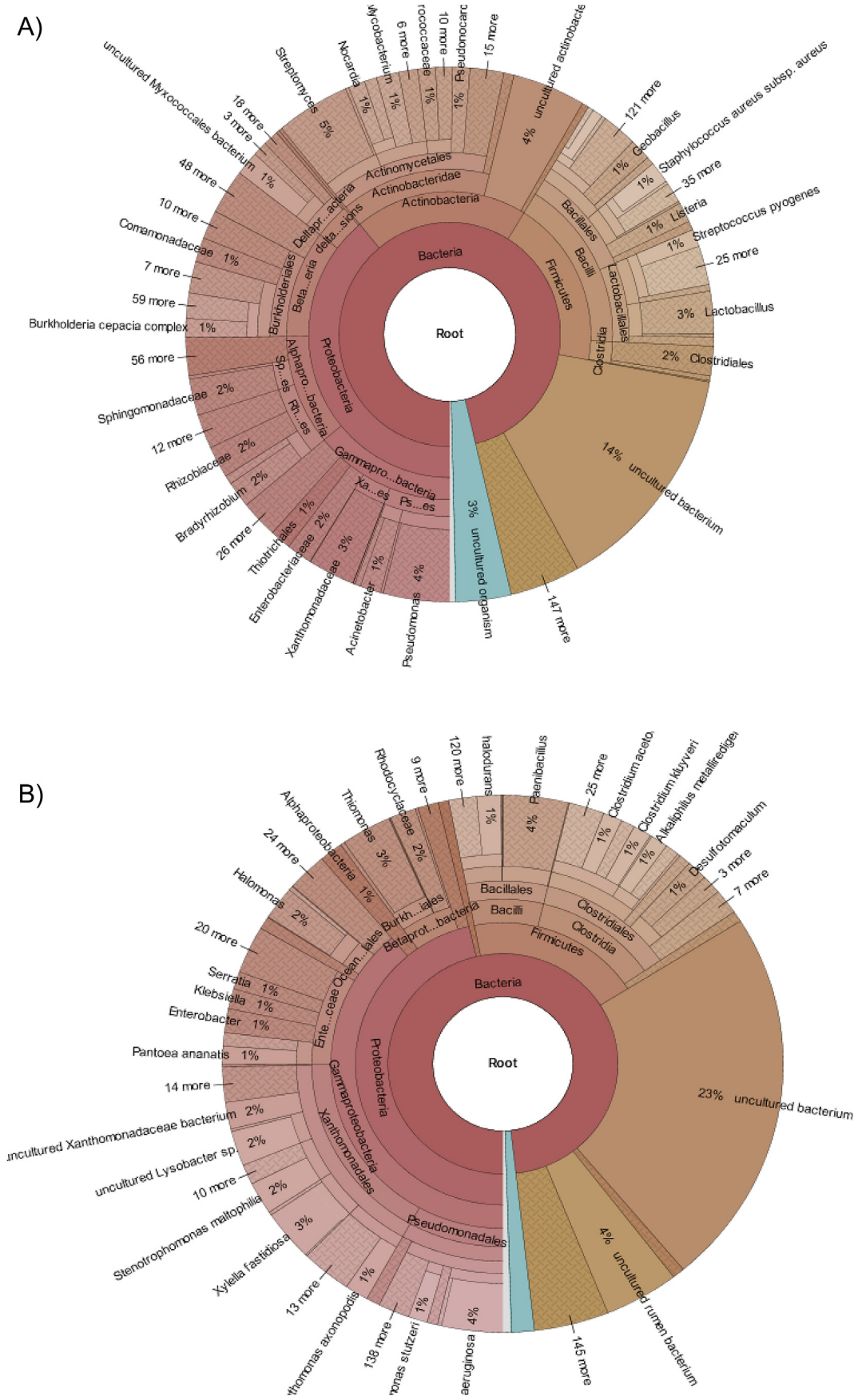


Fig. 3. Taxonomic assignment of T-RFLP profiles. Krona representation of the different bacterial classes sorted out after MiCA matching of T-RFLP profiles obtained with *AluI* (A) and *TaqI* (B) restriction enzymes. Codes are those reported in the legend of Fig. 1.

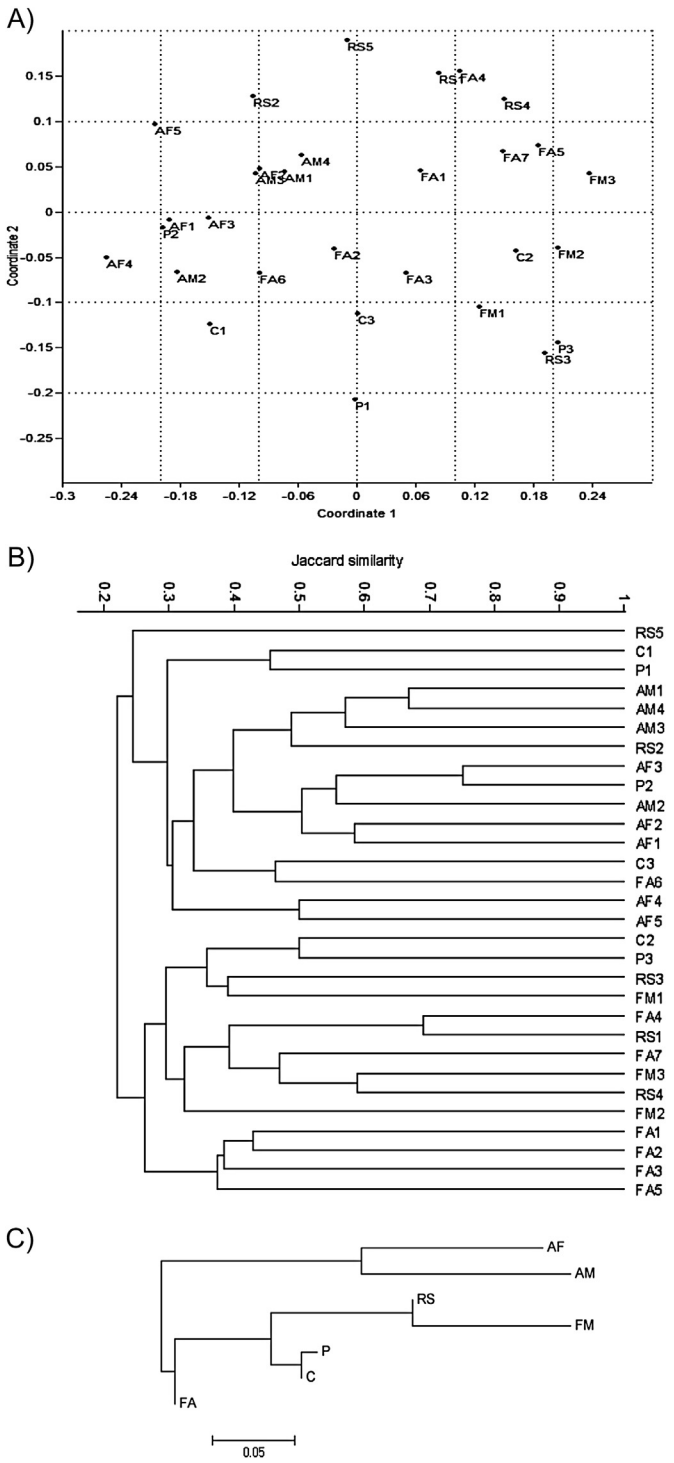


Fig. 4. Differences of bacterial communities. A) Non-Metric Multidimensional Scaling (n-MDS); stress = 0.3013. B) UPGMA dendrogram. Both analyses are based on Jaccard distance matrix between T-RFLP profiles. C) Neighbor Joining dendrogram of Slatkin's linearized pairwise F_{ST}. Codes are those reported in the legend of Fig. 1.

In the present report, the microbiomes of individuals of *Talitrus saltator* were characterized by T-RFLP analysis attempting to explore microbiome composition in this supralittoral crustacean. We showed a high diversity of the microbiome, in agreement (and in some cases higher) with several previous studies on animals (Haynes et al., 2003; Disayathanoowat et al., 2011), and apparently not associated with organic carbon content of sandy sediments and nutritional status of

Table 3
T-RFs accounting for population differentiation after locus-by-locus AMOVA and putative taxonomic groups.

TRF-size*	Putative taxonomic assignment†
AluI	
64	*Spirochaetia* (AY369252; *Treponema vincentii* OMZ 861
82	*Firmicutes* (AM990996; *Bacillus subtilis* subsp. *subtilis* RSP-GL)
160	*Firmicutes* (AY854364; uncultured bacterium Thompsons86. Best Blast hit to NR_028721; *Heliorestis daurensis* with 100% identity, total score 1444)
TaqI	
41	*Gammaproteobacteria* (GU434689 *Pseudomonas stutzeri*; HM438059 uncultured *Oceanospirillales* bacterium; DQ185604 *Klebsiella pneumonia*; FM163485 *Serratia marcescens*; HM438484 uncultured *Halomonas*)
42	*Betaproteobacteria* (AY277699; Candidatus *Burkholderia verschuerenii*; Y14701; *Azoarcus anaerobius*; X77679; *Azoarcus evansii*; AF229881; *Thauera aromatica*; AF229887; *Thauera chlorobenzoica*)
45	No matches after MiCA search
47	*Sphingobacteriia* (X91814; *Sphingobacterium comitans*) *Cytophagia* (GQ161990; *Pedobacter*; EU682684; *Adhaeribacter terreus*; AM230484; *Terrimonas ferruginea*; FJ890894; *Chitinophaga*)
48	No matches after MiCA search
53	*Deltaproteobacteria* (DQ646304; uncultured *Myxococcales* bacterium)
59	*Clostridia* (AY821870; uncultured *Mogibacterium*) *Flavobacteriia* (AY987349; *Nonlabens tegetincola*) *Alphaproteobacteria* (HM438600; uncultured *Sphingomonas*) *Nitrospira* (X71838; Candidauts *Magnetobacterium bavaricum*, HM454280; uncultured *Nitrospirae* bacterium)
70	*Gemmatimonadetes* (AP009153; *Gemmatimonas aurantiaca*)
85	No matches after MiCA search
118	No matches after MiCA search

* The TRF size (nt) is displayed. The name of the restriction enzyme is also reported above the.

† Taxonomic groups displayed are those sorted out after MiCA search over the Ribosomal Database. For each T-RF the name of the class is reported and, in parentheses, the matching sequence with GenBank ID and strain name. For sequences coded as "uncultured" the best Blast hit is also displayed.

populations (estimated as lipid content). It is of note that *T. saltator*-associated bacterial communities contained taxa both known to include members of animal associated species (e.g. *Gammaproteobacteria* and Firmicutes of *Bacillales* and *Clostridiales* classes), but also other classes, such as *Alphaproteobacteria*, which include marine (Morris et al., 2002) and plant-associated species (Ettema and Andersson, 2009). The assignment of TRFs to members of *Clostridiales* may also suggest an involvement of this group of bacteria in cellulose degradation within the gut, as previously reported (Nuti et al., 1971; Martineti et al., 1995), similarly to other herbivores (Zhu et al., 2011). The presence of *Alphaproteobacteria* may be linked to both the interaction of animals with seawater (since they live on the damp band of sandy beaches) and to the feeding of organic debris.

Another important feature was the high variability of microbiome T-RFLP fingerprints among individuals, even from the same populations. In this respect, the inter-individual differences accounted for up to 88.70% of total fingerprints variance. It was interesting to note that statistically significant population-specific microbiome signatures also were detected, and accounted for the remaining 11.30% of total fingerprints variance. These population-specific differences were attributed to several taxa among which the classes of *Gammaproteobacteria*, *Betaproteobacteria*, *Spirochaetia*, *Firmicutes*, *Sphingobacteriia*, *Deltaproteobacteria*, and *Gemmatimonadetes* which contain species known to be associated with eukaryotic organisms and aquatic

invertebrates (see for instances (Harris, 1993; Archie and Theis, 2011)). Finally, a locus-by-locus AMOVA indicated the presence of a fraction of T-RFLP profiles related with animal populations, suggesting a potential role of site or of animal's genotype (e.g. immune systems) in shaping the bacterial community composition.

In conclusion this study showed that a high taxonomic variability is present in the microbiome associated with *Talitrus saltator* as well as a large inter-individual microbiome variation. Future studies focusing on specific organs (e.g. gut) and on the modification of the microbiome in relation to environmental parameters are needed to clarify the trophism and extend to the associated bacterial communities the bioindicator features of *T. saltator*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ecss.2013.08.011.

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